

Effect of age and biopsy site on extracellular matrix mRNA and protein levels in human kidney biopsies

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Background. Investigation of the prognostic value of the expression of mRNA for extracellular matrix (ECM) components with respect to deterioration of kidney function in patients with renal disease requires an evaluation of the basal expression of ECM mRNA in healthy individuals and of the reliability of ECM mRNA measurements. In the current study, the collagen $\alpha 1(\text{IV})/\text{GAPDH}$ (C4:G) and collagen $\alpha 1(\text{I})/\text{GAPDH}$ (C1:G) mRNA ratios and the accumulation of collagen IV and collagen I protein were investigated in renal cortices of individuals of various age. Furthermore, we examined whether the C4:G mRNA ratio measured in a renal biopsy is representative of that in the rest of the kidney.

Methods. To investigate the effect of age on collagen expression, kidneys obtained at autopsy from patients with a normal renal function ($N = 18$; age 19 to 92) were used. C4:G and C1:G mRNA ratios were measured by real-time polymerase chain reaction (PCR) analysis. Accumulation of collagen IV and collagen I protein was measured by quantitative image analysis on immunohistochemically stained sections. To determine whether the site at which a biopsy is taken affects the C4:G mRNA ratio, this ratio was measured in cortical biopsies taken from different locations from each of four kidneys: one without renal disease, one with diabetes mellitus type I, and two with diabetes mellitus type II. C4:G mRNA ratios were measured by using real-time PCR.

Results. The C4:G mRNA ratio, but not the C1:G mRNA ratio or collagen IV protein accumulation, increased significantly with age ($r = 0.55$, $P < 0.03$). Collagen I protein accumulation increased with age ($r = 0.85$, $P < 0.001$) and correlated with the extent of interstitial fibrosis ($r = 0.50$, $P < 0.05$). The C4:G mRNA ratio did not differ significantly within a kidney.

Conclusions. This report shows, to our knowledge for the first time, that in the aging, normally functioning human kidney, there is a dissociation between the levels of mRNA for collagen IV and collagen I and the accumulation of these proteins. The levels of mRNA for collagen IV in a single renal biopsy can be regarded as representative of those in the rest of the kidney.

Key words: collagen, digital image analysis, diagnostic technique, progressive renal insufficiency, kidney injury, interstitial fibrotic lesion.

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These observations should be taken into account when ECM mRNA levels are used for diagnostic purposes.

Injury to the kidney can lead to chronic progressive renal insufficiency, resulting in glomerulosclerosis and interstitial fibrosis, accompanied by excessive accumulation of extracellular matrix (ECM) components. Type IV collagen is one of the main components of both normal and diseased glomerular and tubulointerstitial ECM [1–3]. The expression of type I collagen is low in normal kidney tissue, but high amounts of this collagen are present in interstitial fibrotic lesions [2]. Several studies of fibrogenesis in renal disease have focused on the role of the $\alpha 1$ chains of the two types of collagen. In both human and experimental animal models, it has been shown that the amount of collagen $\alpha 1(\text{IV})$ mRNA [4–6] and collagen $\alpha 1(\text{I})$ mRNA [4, 7, 8] is significantly higher in diseased than in normal tissue. Experiments with animal models have shown that an increase in the levels of mRNA for several ECM molecules in an early stage of renal disease precedes and predicts the development of glomerulosclerosis [4, 5, 9, 10] and interstitial fibrosis [8], thereby potentially allowing early therapeutic intervention [5, 11].

Therefore, it is of clinical importance to investigate the predictive value for disease progression of levels of mRNA for collagen and other ECM components, relative to the accumulation of these proteins, in human kidney biopsies. To do this, information is needed about the basal expression of ECM molecules, which may be age-dependent and heterogeneous in the kidney. Our study addressed two issues: (1) whether the levels of mRNA for collagen IV and collagen I and the accumulation of these proteins change with age in autopsy kidneys, using real-time and digital image analysis of immunohistochemically stained sections; and (2) whether collagen $\alpha 1(\text{IV})$ mRNA levels in glomerular and tubulointerstitial tissue of renal biopsy specimens are representative of those in the rest of the kidney.

METHODS

RNA isolation

In all experiments, RNA from cortical tissue was isolated with 1 mL TRIzol® (GIBCO BRL, Grand Island, NY, USA) per 50 to 100 mg of tissue, as described in the manufacturer's manual. RNA from microdissected glomeruli was extracted with an NP40-based solution, according to a previously described protocol [12]. RNA from microdissected tubulointerstitium was isolated with the RNeasy® mini kit (Qiagen, Chatsworth, CA, USA). RNA isolation procedures were carried out further according to the manufacturer's manual.

cDNA synthesis

In all experiments, 1 to 2 µg of RNA was used for cDNA synthesis. In the reverse transcription (RT) reactions, we used a mixture containing 10 U of avian myeloblastosis virus-reverse transcriptase (Boehringer Mannheim, Mannheim, Germany), 1 µmol/L oligo(dT)₁₅ (Boehringer Mannheim), 2 mmol/L dNTP, 20 U of rRNasin (Promega, Madison, WI, USA), and 1 × RT buffer (Boehringer Mannheim).

Real-time polymerase chain reaction

mRNA levels for collagen α1(IV), collagen α1(I), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time polymerase chain reaction (PCR; Perkin-Elmer ABI Prism 7700™). The forward primer, reverse primer (GIBCO BRL), and TaqMan® probe (Perkin-Elmer Biosystems, Norwalk, CT, USA) for each molecule were 5'-CCT CAA GGG CTC CAA CGA G-3', 5'-TCA ATC ACT GTC TTG CCC CA-3', and 5'-ATG GCT GCA CGA GTC ACA CCG GA-3' [collagen α1(I)]; 5'-ACT CTT TTG TGA TGC ACA CCA-3', 5'-AAG CTG TAA GCG TTT GCG TA-3', and 5'-AAT GGC GCA CTT CTA AAC TCC TCC AGG CAG G-3' [collagen α1(IV)]; 5'-TTC CAG GAG CGA GAT CCC T-3', 5'-CAC CCA TGA CGA ACA TGG G-3', and 5'-CCC AGC CTT CTC CAT GGT GGT GAA-3' (GAPDH). The probe sequence for each transcript was chosen over an exon-intron junction in order to prevent amplification of genomic DNA. The 5' ends of the collagen probes were labeled with the reporter dye tetrachloro-6-carboxyfluorescein (TET), and the 5' end of the GAPDH probe was labeled with 6-carboxyfluorescein (FAM). The 3' ends of all probes were labeled with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The PCR reaction contained 100 nmol/L (GAPDH) or 80 nmol/L (collagen) probe, 300 nmol/L both primers, and TaqMan® Universal PCR Master Mix (Perkin-Elmer), including 300 µmol/L dNTP, 2.5 mmol/L MgCl₂, 0.5 U of AmpErase UNG, and 1.25 U of AmpliTaq Gold DNA polymerase. Reactions were carried out in optical 96-well reaction plates covered with optical

caps (Perkin-Elmer). Amplification cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for one minute. The point (designated as C_T value) in which the fluorescence intensity exceeds the standard deviation of the baseline fluorescence is a measure of the amount of cDNA, and thus of mRNA, in the sample. mRNA signals for the different molecules in each sample were standardized against the GAPDH mRNA signal. All measurements were performed in duplicate. Differences between duplicates never varied by more than one C_T value. As standards, each PCR was run on a fivefold dilution range of 2 pg of plasmid containing the appropriate template.

Assay variation of the real-time PCR

Real-time PCR for collagen α1(IV) and GAPDH was performed on four different days in quadruplicate with cDNA obtained from cultured human mesangial cells. Variations in the intra-assay and interassay test results were calculated for the collagen α1(IV) and GAPDH PCR. We also calculated the assay variation in the measurement of the collagen α1(IV):GAPDH (C4:G) mRNA ratio.

Effect of autolysis time on mRNA

Autolysis time was defined as the time between death of the patient and storage of the renal tissue at -70°C. To investigate the effect of autolysis time on the integrity of mRNA, a controlled autolysis experiment was performed with two autopsy kidneys (autolysis times of 7.5 and 7 hours; patients' age of 43 and 19 years). The kidneys were incubated at 4°C. A piece of cortical tissue was resected from each of the kidneys at 0, 2, 4.3, 6.5, 17.3, 24.3, and 48.3 hours and at 0, 1.5, 3, 17.3, 26.5, and 46.5 hours, respectively. The samples were weighed and RNA was isolated. For each time point, levels of mRNA for collagen α1(IV) and GAPDH were measured in duplicate by real-time PCR.

Effect of age on collagen expression

Kidney tissue. This experiment used 18 autopsy kidneys, including the two kidneys used in the autolysis experiment described earlier in this article. The causes of death of the patients were unrelated to kidney function. None of the patients had a history of kidney or kidney-related disease. The kidneys had an autolysis time between 4 and 21 hours. The age of the patients, 11 males and 7 females, varied between 19 and 92 years (mean 58.6 ± 20.8). There was no correlation between autolysis time and age. Periodic acid-Schiff (PAS)-stained sections of the kidneys showed normal glomeruli and glomeruli with global glomerulosclerosis (that is, completely sclerotic glomeruli). For each kidney, the percentage of glomeruli with global glomerulosclerosis of the total amount of glomeruli in the sections were scored. The extent of

interstitial fibrosis also was scored on a scale from 0 to 3+ (0 = no; 1+ = some; 2+ = extensive; and 3+ = severe interstitial fibrosis) and the extent of tubular atrophy on a scale from 1+ to 4+ (1+ = 0 to 10%; 2+ = 11 to 25%; 3+ = 26 to 50%; and 4+ = >50% of the total tubular area). In some kidneys, there was extensive autolytic damage, mainly in the tubulointerstitium, but this was not related to autolysis time or to age. Serum glucose (mean 6.9 ± 2.1 mmol/L), creatinine (mean 89.0 ± 33.5 μ mol/L), and total protein (mean 64.9 ± 10.8 g/L) concentrations were normal. The serum albumin concentration (mean 33.1 ± 9.1 g/L) was slightly below normal. Data of dipstick urine protein were available for nine patients and ranged from negative to 1+. Two patients (aged 63 and 89) had systolic blood pressure values higher than 150 mm Hg and diastolic blood pressures higher than 80 mm Hg.

Experimental setup. RNA was isolated from the cortical tissue of the 18 autopsy kidneys. All RNA samples (1.5 μ g) were converted into cDNA at the same time to limit intersample differences in the efficiency of cDNA synthesis. C4:G mRNA ratios and collagen $\alpha 1(I)$:GAPDH (C1:G) mRNA ratios were assessed in duplicate by real-time PCR. To reduce assay variation, transcripts were measured in one PCR run on the same plate. mRNA measurements for two kidneys were left out of the analysis because of missing data.

For immunohistochemical analysis of collagen IV and collagen I, cortical tissue was snap frozen in precooled isopentane and embedded in OCT compound (Tissue-Tek, Sakura, Zoeterwoude, The Netherlands). Four micrometer cryostat sections were air dried for one hour and fixed with acetone and methanol for 10 minutes. The sections were incubated with a goat anti-human collagen IV antibody or a goat anti-human collagen I antibody (Harlan Sera-lab, Sussex, UK) for one hour, followed by incubation with a FITC-conjugated rabbit anti-goat IgG antibody (Dako, Glostrup, Denmark) for 30 minutes. The sections were incubated in a filtered solution of 0.5 mg/mL diaminobenzidine and 0.02% hydrogen peroxide for 10 minutes, and then in 0.5% $\text{CuSO}_4/0.9\%$ NaCl for 5 minutes, and were counterstained in hematoxylin for 15 seconds. In between the different steps, the slides were washed with phosphate-buffered saline (PBS). For each immunohistochemical assessment, all sections were stained in one session simultaneously. Digital image analysis was performed using a Zeiss microscope equipped with a full-color 3CCD camera (Sony DXC 950p) and KS-400 image analysis software version 3.0 (Zeiss-Kontron, Eching, Germany). Collagen protein accumulation in each section was determined by calculating the percentage of surface stained by the antibody relative to the total surface area in five microscopic fields at 20-fold magnification. Large vessels were not counted.

Effect of biopsy site on C4:G mRNA ratio

Kidney tissue. These experiments used three cadaveric donor kidneys that were not suitable for transplantation because of technical reasons (kidneys 1, 2, and 3) and one autopsy kidney (kidney 4). These kidneys had not been used in the other experiments and were obtained from Eurotransplant. After perfusion, the kidneys were transported on ice to our laboratory. For each of the four kidneys, the percentage of focal and global glomerulosclerosis, the extent of interstitial fibrosis, and the extent of tubular atrophy, were scored as outlined previously in this article.

Kidney 1 (63-year-old male) contained 1.2% focal and global glomerulosclerosis. Interstitial fibrosis and tubular atrophy were virtually absent. Blood glucose, serum creatinine, and urine protein levels were normal. Kidney 2 (61-year-old male) contained 8.9% focal and global glomerulosclerosis. Interstitial fibrosis and tubular atrophy were 1.5+ and 2+ (11 to 25% of the total tubular area), respectively. Blood glucose was 16.8 mmol/L. Serum creatinine and urine protein levels were normal. The patient had diabetes mellitus type I and received insulin therapy. Blood pressure was slightly above normal. Kidney 3 (55-year-old male) contained 9.9% focal and global glomerulosclerosis. The rest of the glomeruli showed some mesangium expansion, reminiscent of that seen in diabetic nephropathy. Interstitial fibrosis and tubular atrophy were 1+ and 2+ (11 to 25% of the total tubular area), respectively. The patient had diabetes mellitus type II and received metformin (Glucophage®) therapy. Blood glucose concentration was 25.2 mmol/L. Serum creatinine concentration was normal, and urine protein concentration was 1.54 g/L. Kidney 4 was obtained at autopsy (69-year-old female; autolysis time of 4 hours). In the glomeruli, 43.7% focal and global glomerulosclerosis was seen. The other glomeruli showed expansion of the mesangium. Interstitial fibrosis and tubular atrophy were 2+ and 3+ (26 to 50% of the total tubular area), respectively. Diabetes mellitus type II was diagnosed one year before death. During the last year of life, the blood glucose concentration increased from 7.8 to 17.9 mmol/L, the urine protein concentration from 1.36 to 2.29 g/24 h, and the serum creatinine concentration from 95 to 329 μ mol/L and then to 510 μ mol/L a few days before death. The patient had a previous history of hypertension.

Accuracy of microdissection. A biopsy of approximately 4 mm long and 1 mm wide was resected from the outer, subcapsular cortices of kidneys 2 and 4. To separate glomeruli from the surrounding tubulointerstitium, microdissection was performed visually under stereo microscopy, as described in a previous report [12]. The biopsy tissue was disrupted with the aid of two needles, and the glomeruli (54 and 50 from the biopsy of kidneys 2 and 4, respectively) were as accurately removed from the

tubulointerstitium as possible. To evaluate the microdissected tubulointerstitium for the presence of any remaining glomeruli, the tubulointerstitial fractions were pelleted by centrifugation for one minute at room temperature and fixed in glutaraldehyde. The fractions were embedded in Spurr plastic and sliced in 1 μm and ultrathin sections with a 200 μm interval. The sections were stained with toluidine blue and evaluated by bright field (1 μm sections) and phase contrast microscopy (ultrathin sections). To test whether the efficiency of dissecting globally sclerotic glomeruli was different from that of dissecting nonsclerosed glomeruli, the percentage of globally sclerotic glomeruli that remained behind in the microdissected tubulointerstitial pellet was compared with the percentage of globally sclerotic glomeruli stained with PAS in the nondissected tissue from different locations in the same kidney (discussed later in this article).

Experimental setup. Five biopsies (3 to 4 mm long and 1 mm wide) were taken at different locations from the outer, subcapsular cortices of kidneys 1, 2, 3, and 4. The biopsies were taken from the following segments of the right kidneys: 1, lower segment, near lower segmental artery; 2, upper segment, near upper segmental artery; 3, lower segment; 4, apical segment; and 5, middle segment. The glomeruli and the tubulointerstitium were separated by microdissection under stereo microscopy in a Petri dish containing ice-cold PBS. The glomeruli were transferred to a second Petri dish containing ice-cold PBS and washed free of surrounding tissue debris. The purity of the glomeruli was determined visually. The glomerular and tubulointerstitial fractions from each biopsy were collected in separate reaction tubes. The number of glomeruli obtained from the biopsies ranged from 20 to 60. mRNA levels for collagen $\alpha 1(\text{IV})$ and GAPDH in the glomerular and tubulointerstitial tissue from each biopsy were measured in duplicate by real-time PCR.

Statistical analysis

In the experiments evaluating the effect of age, correlations between histological data, mRNA data, and immunohistochemical data were analyzed with Pearson correlation tests. Differences in mRNA data and immunohistochemical data between age groups were tested with one-way analysis of variance (ANOVA). In the biopsy-site experiments, differences between values for each of the five biopsies from a kidney and the average of these five values were analyzed by one-way ANOVA with post hoc testing for multiple comparisons. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Assay variation of the real-time PCR

The intra-assay variation of the real-time PCR for collagen $\alpha 1(\text{IV})$ and GAPDH was 16.4 and 11.6%, re-

spectively. The assay variation for the assessment of the C4:G mRNA ratio was 28.8%. This means that differences in C4:G mRNA ratios between samples of less than twofold were due to assay variation and not to real differences in expression.

Effect of autolysis time on mRNA

In the two autopsy kidneys tested, both collagen $\alpha 1(\text{IV})$ mRNA levels ($r = 0.97$ and $r = 0.90$, respectively; Fig. 1A) and GAPDH mRNA levels ($r = 0.87$ and $r = 0.91$, respectively; Fig. 1B) decreased with increasing autolysis time so that the C4:G mRNA ratio did not change when the autolysis time increased ($r = 0.32$ and $r = 0.19$, respectively; Fig. 1C).

Effect of age on collagen expression

The percentage of focal and global glomerulosclerosis ($r = 0.51$, $P < 0.04$), the extent of interstitial fibrosis ($r = 0.53$, $P < 0.04$), and the extent of tubular atrophy ($r = 0.66$, $P < 0.02$) increased with age (19 to 92 years; data not shown). Between 19 and 92 years of age, the C4:G mRNA ratio increased ($r = 0.55$, $P < 0.03$; Fig. 2A), whereas the C1:G mRNA ratio remained constant ($r = -0.13$, $P = \text{NS}$; Fig. 2B). With increasing age, collagen IV protein accumulation did not change ($r = 0.26$, $P = \text{NS}$; Fig. 2C), whereas collagen I protein accumulation increased ($r = 0.85$, $P < 0.001$; Fig. 2D). The mean C4:G mRNA ratio in kidneys from individuals older than 60 years (5.4 ± 3.2) was 3.2 times higher than that in kidneys from individuals younger than 60 years (1.7 ± 0.7 , $P < 0.01$). In these two age groups, the mean C1:G mRNA ratio (2.3 ± 2.8 and 2.9 ± 3.1 , respectively) and the mean accumulation of collagen IV protein ($25.9 \pm 1.8\%$ and $24.4 \pm 5.0\%$, respectively) were not significantly different. The mean accumulation of collagen I protein was greater in kidneys from individuals older than 60 years ($21.0 \pm 3.5\%$) than in kidneys from individuals younger than 60 years ($15.0 \pm 4.7\%$, $P < 0.02$). There was no correlation between the C4:G mRNA ratio and collagen IV protein accumulation or between the C1:G mRNA ratio and collagen I protein accumulation. There were no differences in histology, mRNA expression, and collagen accumulation between kidneys from males ($N = 11$) and females ($N = 7$). We tested correlations between collagen mRNA expression or collagen accumulation and the percentage of focal and global glomerulosclerosis, the extent of interstitial fibrosis, and the extent of tubular atrophy. There were significant correlations between the percentage of focal and global glomerulosclerosis and the C4:G mRNA ratio ($r = 0.52$, $P < 0.04$) and between the extent of interstitial fibrosis and the amount of collagen I protein accumulated ($r = 0.50$, $P < 0.05$; data not shown).

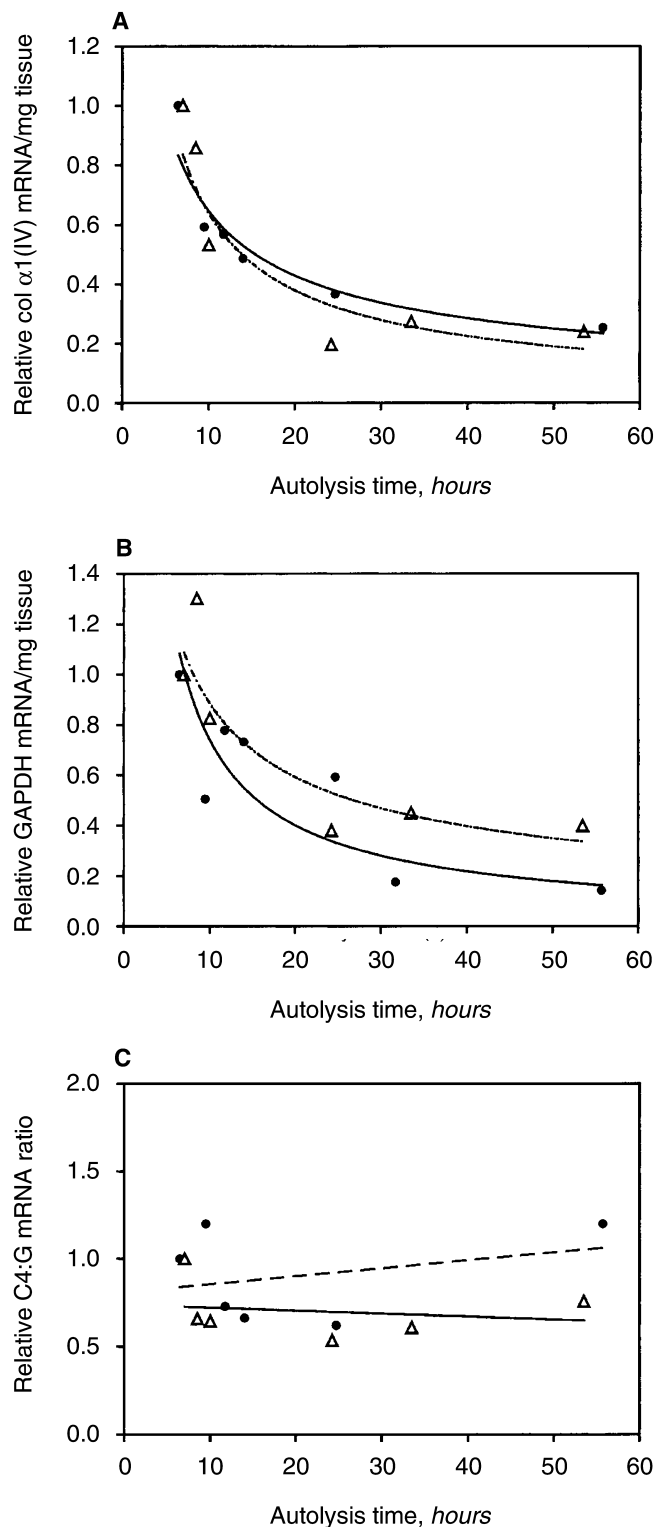


Fig. 1. Effect of autolysis time on collagen $\alpha 1(IV)$ mRNA (A), GAPDH mRNA (B), and the collagen $\alpha 1(IV)$:GAPDH (C4:G) mRNA ratio (C). Two kidneys (●, age 43, autolysis time of 7.5 hours; △, age 19, autolysis time of 7 hours) were obtained at autopsy and immediately incubated at 4°C. At set time intervals, a piece of cortex was removed for measurement of collagen $\alpha 1(IV)$ mRNA, GAPDH mRNA, and the C:G mRNA ratio. Signal at the first time point of each kidney was set to one. Signals at other time points are shown relative to those at the first time point. Data are expressed as means of duplicate measurements per mg tissue.

Accuracy of microdissection

From the biopsy of kidney 2, 54 glomeruli were dissected. Eight glomeruli remained behind in the tubulointerstitial fraction (12.9% of the total number of glomeruli). One of these glomeruli (12.5%) was globally sclerotic, a percentage consistent with the extent of focal and global glomerulosclerosis (range 4.3 to 13.5%, mean $8.9 \pm 3.7\%$) detected at different locations in the corresponding kidney.

From the biopsy of kidney 4, 50 glomeruli were dissected, and 12 remained behind in the tubulointerstitium (19.4%). Five of the 12 glomeruli (41.7%) were globally sclerotic, a percentage consistent with the extent of focal and global glomerulosclerosis (range 33.3 to 53.8%; mean $43.7 \pm 8.6\%$) detected at different locations in the corresponding kidney.

Effect of biopsy site on C4:G mRNA ratio

Figure 3 shows the C4:G mRNA ratios measured by real-time PCR in the glomerular and tubulointerstitial compartments of five biopsies taken from different locations in each of the four kidneys tested (A through D). The C4:G mRNA ratio of individual biopsies did not differ more than twofold (as determined in the assay variation experiment) from the average ratio for the five biopsies. Analysis of variance showed no significant differences ($P > 0.05$). We concluded that C4:G mRNA ratios in a single biopsy were representative of those in the rest of the kidney.

DISCUSSION

Analysis of expression of ECM components is an important step in unraveling the molecular basis of the development of human renal disease. Collagen IV and collagen I are major components of the ECM, and their synthesis is up-regulated in diseased kidney tissue [4–6, 8]. We therefore measured the expression of these molecules in autopsy kidneys to determine whether their expression is age related and whether levels of collagen IV mRNA measured in microdissected, glomerular, and tubulointerstitial tissue from a biopsy are representative of levels of collagen IV mRNA at other sites in the kidney.

The assay variation in C4:G mRNA measurements was 28.8% and those for the measurements of collagen IV mRNA and GAPDH mRNA were 16.4 and 11.6%, respectively. This is in accordance with our observations that duplicate measurements of a transcript in any sample show a difference that roughly is in the same order of magnitude.

Then, the effect of autolysis time on the integrity of mRNA was investigated. In controlled autolysis experiments with kidneys kept at 4°C, the levels of both collagen IV and GAPDH mRNA gradually decreased between 7.5 and 56 hours postmortem. These findings are

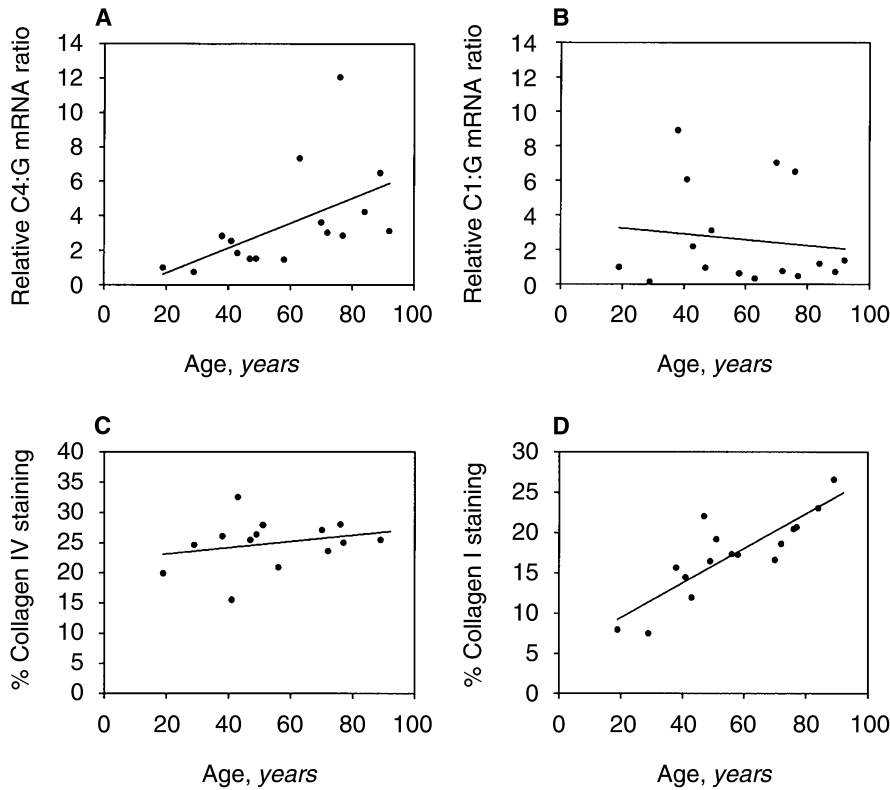


Fig. 2. Effect of age (A) on the collagen $\alpha 1(\text{IV})/\text{GAPDH}$ (C4:G) mRNA ratio and (B) on the C1:G mRNA ratio. The C4:G mRNA ratio and C1:G mRNA ratio in cortical tissue from kidneys taken from healthy individuals (19 to 92 years old) at autopsy were assessed by real-time PCR. Each panel shows relative mRNA ratios to the mRNA ratio in the youngest kidney. Data are presented as means of duplicate measurements. Effect of age (C) on collagen IV accumulation and (D) on collagen I accumulation. Four micrometer cryostat sections from cortical tissue of the autopsy kidneys were immunohistochemically stained with collagen IV antibodies and collagen I antibodies, respectively. The surface area stained was quantified by digital image analysis at $\times 20$ magnification. Results for each kidney are shown as the percentage of surface staining relative to the total surface area of five microscopic fields in a section.

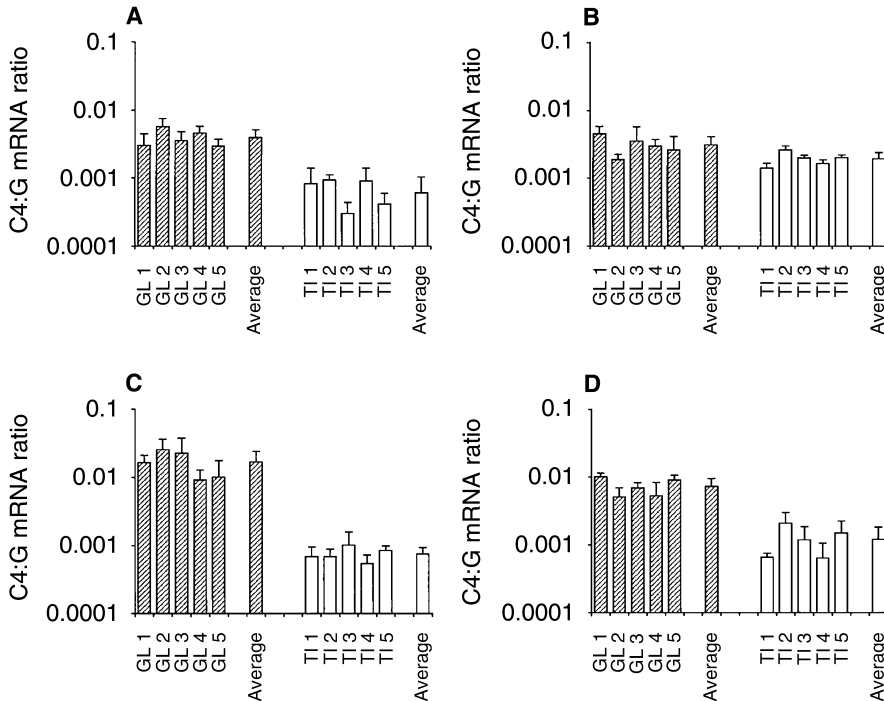


Fig. 3. C4:G mRNA ratio in biopsy specimens from different sites of the renal cortex. Four kidneys were used for this experiment. (A) Kidney 1 was from a patient with normal renal parameters (blood glucose, serum creatinine, urine protein). (B) Kidney 2 was from a patient diagnosed with type I diabetes. Kidneys 3 (C) and 4 (D) were from patients diagnosed with type II diabetes. Of each kidney, five biopsies were taken from the cortex in different locations of the kidney. Glomeruli (GL; \square) and tubulointerstitium (TI; \square) were separated by microdissection. The C4:G mRNA ratio was assessed by using real-time PCR. The panels show data for the individual biopsies and the average of these data (avg). Data are expressed as means \pm SD.

in contrast with those from other studies in which mRNA signals for several transcripts, measured by Northern blot analysis in lung [13] and brain tissue [14], were found

to be similar at all time points up to an autolysis time of 48 hours at 4°C . These contrasting results may be explained by differences in the stability of the mRNA

transcripts that were measured, the sensitivity of the techniques used for mRNA quantitation, the tissues investigated, or a combination of these factors. The C4:G mRNA ratio remained unaltered with increasing autolysis time. On the basis of this finding, we concluded that it was justified to use the C4:G mRNA ratio to investigate the effect of age on the expression of collagen IV mRNA in autopsy kidneys.

Levels of mRNA for collagen IV increased with age. This is in accordance with results from a previous study with rats [15]. However, levels of mRNA for collagen I did not change with age, which is in contrast with previous findings for rats [15, 16]. We corrected the mRNA signal of both collagens for the mRNA signal of GAPDH. There was a positive correlation between collagen IV mRNA yield per mg tissue with age, but not between collagen I mRNA yield per mg tissue or GAPDH mRNA yield per mg tissue and age. This indicated that the increase in the C4:G mRNA ratio in older kidneys was not due to a decrease in GAPDH mRNA yield per mg tissue, but rather to an increase of collagen IV mRNA yield per mg tissue.

The amount of collagen IV and collagen I protein was quantified by digital image analysis, an accurate and reliable method for quantification of protein accumulation in renal tissue [17]. Our finding that collagen I protein accumulation increased significantly with age was consistent with results from another study that showed elevated levels of collagen I protein, measured by Western blotting, in renal cortices of aging rats [15]. Furthermore, in our study, collagen I protein accumulation correlated with the extent of interstitial fibrosis, indicating that collagen I was a key component in the age-related development of these interstitial fibrotic lesions. Collagen IV protein accumulation did not change with age and thus deposition of this collagen did not contribute to the development of interstitial fibrotic lesions. The observation that collagen IV protein accumulation is not age related is consistent with results from previous studies. For example, Abrass, Adcox, and Raugi showed that collagen IV accumulation did not increase in kidneys from aging rats [18]. In their study, collagen I accumulation increased slightly in focal areas of interstitial fibrosis only. In another study of human tissue, the amount of collagen IV protein, measured as the 7-S domain, in the renal cortex did not increase significantly between 30 and 90 years of age [19].

The observation that between the ages of 19 and 92 years the level of mRNA for collagen IV and collagen I and the accumulation of these proteins did not change in parallel might point to a change in the turnover of these collagens in the aging kidney. This could result from changes in the activity of different matrix metalloproteinases (MMPs) responsible for the catabolism of collagen. Whereas collagen IV is mainly degraded by

stromelysins (such as MMP-3) and gelatinases (such as MMP-9), collagen I is mainly degraded by interstitial collagenases (such as MMP-8 and MMP-13) [20, 21]. Possibly, during aging, the expression and activity of collagen I-recognizing MMPs change differently from those for collagen IV-recognizing MMPs, or the efficiency of translation of the collagen transcripts is altered in the aging kidney. It is also conceivable that post-transcriptional modifications or altered polymerization and incorporation of collagen in the ECM are responsible for either an increased or reduced susceptibility to protein catabolism.

We found the C4:G mRNA ratio measured in biopsy samples taken from different sites in the cortex of kidneys with various histological changes to be representative of those for the entire kidney. There were no significant differences in C4:G mRNA ratios in biopsies taken from different locations in the kidney, and thus, the C4:G mRNA ratio for a biopsy sample is representative of the C4:G mRNA ratio for the whole kidney. Our results showed that the C4:G mRNA ratio in the glomerular compartments was relatively higher in kidneys with diabetic nephropathy (kidneys 3 and 4) than in non-nephropathic kidneys (1 and 2). This implies that the level of mRNA for ECM components may be of diagnostic value in patients with renal disease. It would be intriguing to investigate mRNA levels for collagen and other molecules assumed to be involved in the development of renal disease in kidneys with additional glomerular and tubulointerstitial disorders.

To our knowledge, this study is the first to show the application of real-time PCR to measure mRNA in the glomerular and tubulointerstitial compartments of the human renal cortex. The results described provide a number of practical considerations that must be taken into account when quantifying ECM mRNA levels in human biopsy material and when using these levels for diagnostic and prognostic purposes.

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